Histomorphometric changes in the wing bones of the fruit bat, *Pteropus poliocephalus*, (Megachiroptera: Pteropidae) in relation to increased bone strain and the failure of a good (?) hypothesis

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ABSTRACT

Fluorochrome labelling of bone formation was used to examine the effect of exercise (flight) on the wing skeleton of fruit bats, *Pteropus poliocephalus*, over a 194 day period. The bats in this study had been born and raised in captivity and it was hypothesised that the large increases in bone strain that accompanied active flight would result in bone formation at the periosteal bone surface, leading to increased mechanical stiffness and strength. This hypothesis was not supported by the results. Bone formation rates, percentage mineralising surface and mineral apposition rates at the mid-shaft periosteal surface of the radius, metacarpal III and metacarpal V were small. The proximal phalanx of digit V did not display any bone formation at this surface. Bone appositional activity was not significantly different between baseline, control (non-flight) and treatment (flight) groups at any time-point of the experiment. Apposition, although limited, occurred primarily at the endocortical surface in all bones of all animals. No correlation was found between activity and bone formation. Active intracortical remodelling (a total of four secondary osteons) was only seen in three individuals. There was evidence of earlier remodelling activity in most bones, although there was no evidence of any secondary remodelling in the proximal phalanx.

Key words: Exercise, Flight, Fluorochrome, Apposition, remodelling, grey-headed flying-fox.

Introduction

The grey-headed flying-fox, *Pteropus poliocephalus*, is a pteropid fruit bat endemic to Australia, growing to a body mass of about one kilogram. Its large size and availability allowed Swartz *et al*., (1992) to examine bone deformations during unrestrained active flight, using rosette strain gauges bonded to the humerus and radius. Analysis of strain data showed these bones to be exposed to longitudinal stresses of magnitudes comparable to those recorded in the long bones of terrestrial mammals during strenuous exercise (Rubin & Lanyon, 1982; Biewener *et al.,* 1983). However, significant shear stresses of more than 15 megapascals, indicative of substantial torsional loads, were also found in these bones. It was proposed that these torsional (supinating) forces occur in vertebrates capable of active, muscle-powered flight due to the wing skeleton lying anterior to the centre of aerodynamic lift.

This interpretation is consistent with observations of relatively large diameters and thin cortices occurring in proximal wing bones of bats, birds and pterosaurs (Currey & Alexander, 1985; Swartz *et al*., 1992; van der Meulen *et al*., 1992). This morphology may be a derived condition that evolved with flight, as it maximises bone strength and stiffness for any given bone mass, which is presumably advantageous for flying animals.

It is known that long bones of mammals respond to their mechanical environments, particularly during their initial growth (Biewener & Bertram, 1993). Low bone strains, such as encountered in conditions of non-weight bearing or immobilisation, result in a net loss of bone. Under such circumstances activation of intracortical remodelling results in a proposed increase in bone turnover, endocortical bone loss accelerates by modelling events whilst periosteal bone apposition may be retarded. Conversely, high bone strains are generally linked to periosteal bone formation, retardation of intracortical remodelling and, either a reduction in the rate of bone loss or bone formation at the endocortical surface (Jones *et al*., 1977; Rubin & Lanyon, 1985; Martin & Burr, 1989, Turner *et al*., 1994).

This present study primarily aimed to investigate mid-shaft cortical bone responses to increased levels of strain in the wings of *P. poliocephalus*. The subject animals were non-flying bats from a captive colony that were relocated to a large cage and trained to fly. Bone formation was followed over a seven-month period using fluorochrome labelling. We tested the hypothesis that there would be an adaptive, anabolic response of wing bones when they were exposed to elevated flight-induced strains. Bone apposition was predicted to occur at the periosteal surface as this is the most biomechanically relevant site for increasing bone stiffness and strength. The relative

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mineralisation of skeletal elements from free-flying fruit bats was also explored, to assist in the interpretation of fluorochrome label intensity in the captive bats.

Materials and Methods

Wild-caught bats

Wing bone density was determined for the femur, humerus, radius, metacarpal III and IV, proximal phalanx III and IV, and distal phalanx III and IV in seven wild *P. poliocephalus*. A mid-shaft section of each bone was defatted in a methanol/chloroform (2:1 v/v) mix, weighed in air and when suspended in water, with its density determined by Archimedes' principle.

Captive bats

Twelve, three year old grey-headed flying foxes, *Pteropus poliocephalus*, (body mass, M_B = 751 ± 83 g, mean and standard deviation) were obtained from a captive colony maintained at the University of Queensland Veterinary Farm, Pinjarra Hills. These bats were born and raised in a cage (1.9 m high x 3 m x 7 m) constructed of wire-link fencing. Bats would move around the cage by suspensorial quadrupedal locomotion and did not fly. Radiographs of each animal showed that all long bone epiphyses were closed, confirming that they were skeletally mature prior to the start of this study.

Bats were placed in either a baseline (3 animals), control (4) or flight group (5). Two animals had small defects to the wings and were therefore placed in either the control or baseline groups. On day zero, each animal was given alizarin complexone (20 mg.kg M_B^{-1}, Sigma Chemical Co.) made up in sterile 2% $Na(CO_3)_2$, 0.9% NaCl, pH 7.45, via subcutaneous injection in the interscapular region. Two doses were administered 10 h apart. This regimen was repeated on day 12. Baseline animals were euthanased with 1 ml Lethabarb (Sodium pentabarbitone, 500 mg.ml^{-1}, i.p.) on day 15. Up to this stage all animals were maintained in their normal holding cage (see above). The control group of bats was then moved and housed in a 2 m x 2 m x 2 m cage for 194 days and were supplied with fresh fruit (paw paw, mangoes, custard apples, apples, cherries, nectarines, grapes, melons), a salt lick and water containing vitamin and calcium supplements *ad libertum*. The flight group was treated similarly, except for an exercise period each day during which they were moved into a 30 m x 2 m x 2 m flight cage. Individual bats were carried by hand to the far end of the flight cage, relative to their feeding and housing cage, and released from a height of 1.5 m (Swartz *et al*., 1992). They would fly the length of the cage and land on hessian sacking suspended from the walls. Bats were encouraged to fly 180 m per day, taking about 120 wing beat cycles to cover this distance. All bats were given further fluorochrome bone markers (all from Sigma Chemical Co.) as follows; Calcein (5 mg.kg M_B^{-1}) – day 65 and 76, Xylenol orange (60 mg.kg M_B^{-1}) – day 129 and 140, Tetracycline HCl (10 mg.kg M_B^{-1}) – day 196 and 206. Three days after the last label was given all animals were euthanased. Bats were re-radiographed and weighed. The wing and hind limb bones were removed and fixed for 24 h in 10% neutral buffered formalin. Subsequent dehydration, through an ethanol series and xylene, was followed by infiltration and embedding in methymethacrylate. Transverse sections (50 μm) were cut with a diamond-wire saw (Ahlburg Technical Equipment Corp., GA) from the mid-diaphysis of the radius, metacarpals III and V, and the proximal phalanx of digit V. Sections were washed in distilled water, sonicated, dehydrated through to xylene, mounted on a microscope slide with Eukitt (Kindler, Germany) and a cover slip. Sections were examined under a Nikon Optiphot-2 microscope with normal illumination or with reflected ultraviolet light to view the fluorochrome labels.

Measurements were made using the Bioquant semiautomatic digitising system (R&M Biometrics, TN). The following histomorphometric parameters were examined for both periosteal (Ps) and endocortical (Ec) surfaces in each bone mid-shaft section (Li *et al*., 1991). *Mineral apposition rate* (MAR) = The rate of bone apposition (μm/day) determined from the mean distance between the two fluorescent lines produced by each of the fluorochrome labels used and the interlabel period. *Mineralising surface* (MS) = The measured lengths of the lines of fluorescently labelled bone (Double label length + 0.5·single label length) for each fluorochrome label, expressed as a percentage of the relevant bone surface (BS). *Bone formation rate* (BFR) = MAR x MS/BS ($\mu m^3/\mu m^2$/yr). *Eroded surface* (ES) = Erosion (absorption) perimeter as a percentage of the total surface perimeter.

Additional 100 μm mid-diaphysial cross-sections of the radius and metacarpal V were taken from sites adjacent to those used for histomorphometry. Digital images were collected of each cross section (BH-2 microscope, Olympus; Spot 1.4.0. digital camera, Diagnostic Instruments Inc.). Cortical area (*A*), second moment of area (*I*), polar moment of inertia (*J*) and morphology in terms of bone radius/cortical thickness (Currey & Alexander, 1985) were determined (NIH image software).

Muscle masses for 41 muscles from the fore limb, cervical and thoracic regions were measured (± 0.1 mg, Sartorius A200S) for all bats from each of the treatment groups.

Statistical Analysis

Data are presented as mean and standard error unless otherwise stated. Student's *t*-test was used to examine differences between non-flight (control) and flight groups at the different time points. A one-way analysis of variance (ANOVA) was used to examine whether differences in density occurred between different bones in the wild-caught bats. The Student-Newman-Keuls method was applied *post hoc* to determine where differences lay. Significance was assumed if $P < 0.05$.

Results

General Observations

All bats held in captivity remained in good health for the duration of this project. The average mass of the bats in each group was; for the baseline group 738 ± 137.4 g, the control (non-flight) group 743 ± 65.3 g, and the treatment (flight) group 766 ± 76.3 g. The final mass on day 209 was 778 ± 123.1 g for the non-flight group and 713 ± 90.2 g for the flight group.

Flight Ability

Animals in the treatment group were unable to fly more than a few wing beat cycles at the beginning of the experimental period. Encouragement in the form of a food reward (pieces of Mars bar, Cadburys) coupled with repeated short distance (c. 5 m) training flights resulted in the bats quickly developing the capability to fly six lengths of the flight cage (180 m.day^{-1}, 120 wing-beat cycles). An equivalent amount of 'reward food' was fed to control animals.

Fluorochrome Labelling

Alizarin, calcein and xylenol labels were present in all bats to which they were administered, but the tetracycline label was not observed in any animals. It is assumed that the application of this fluorochrome was unsuccessful.

Radius:

The level of bone formation in the captive population at the start of the experiment was low. Only six of the 12 bats had the alizarin label associated with the periosteal surface, with an average mineralising surface (Ps MS/BS) of 0.6 ± 0.27 %. The endosteal surface was more active with all baseline and flight group bats showing considerable labelling (Fig. 1a). However, none of the control group had alizarin label showing at the endosteal surface.

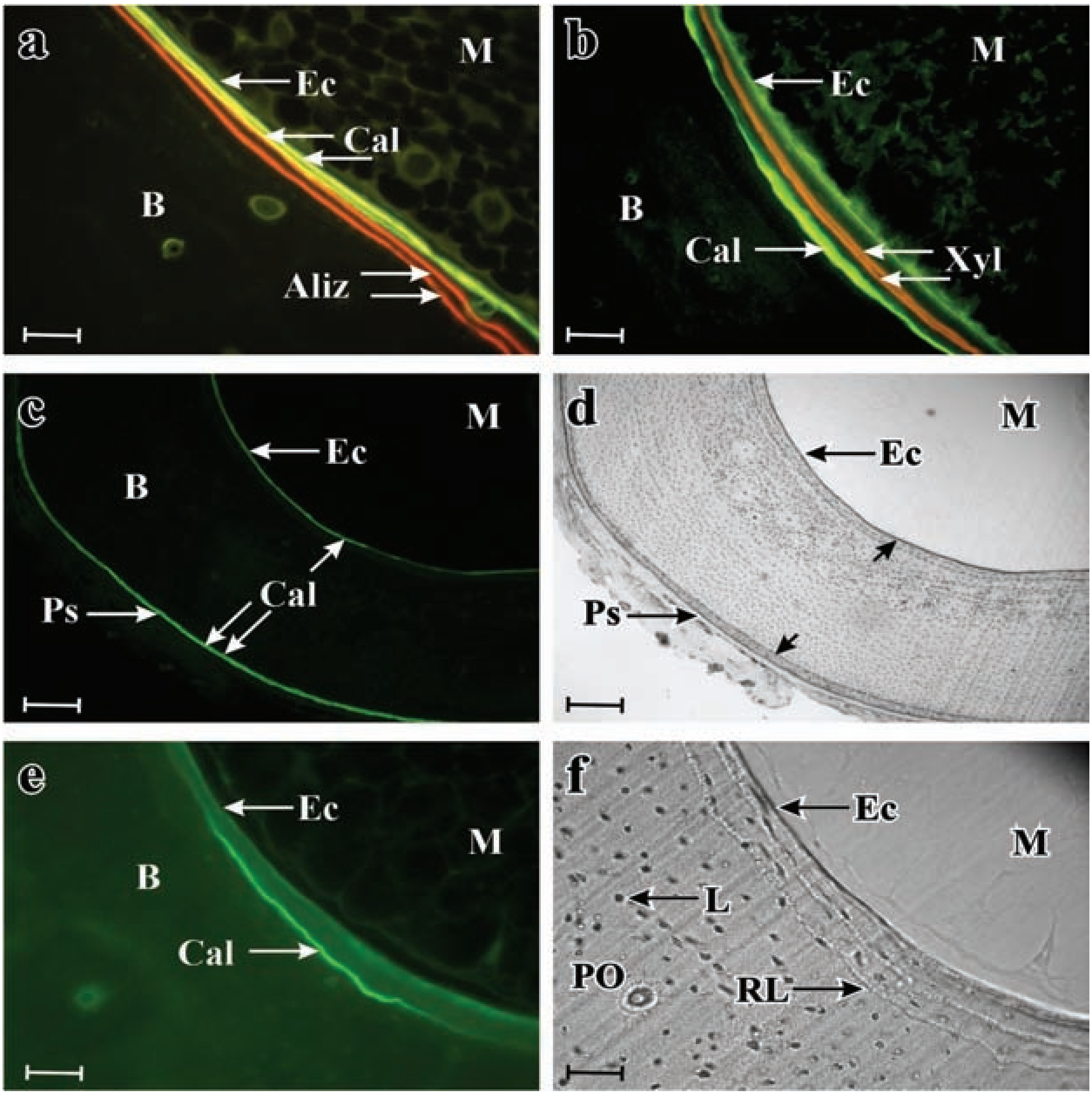

Figure 1. Mid-shaft transverse sections of undecalcified wing-bones of *Pteropus poliocephalus.* (a), (b) Fluorochrome labelling of bone apposition at the endocortical (Ec) surface of the radii in two bats. Regions of double and single labelling with alizarin (Aliz), calcein (Cal) and xylenol (Xyl) can be seen within the bone (B). Bone marrow (M) is indicated in the upper right quadrant of all pictures. (c) The full cortical width of a radius showing calcein labelling at both the periosteal (Ps) and endocortical sufaces. (d) Light micrograph of (c) showing bone internal structure. Arrows indicate the position of the incorporated calcein label. (e) Single calcein label in a fifth metacarpal. (f) Light micrograph of (e) showing a primary osteon (PO), lacuna (L) and reversal line (RL). Scale bars: (a), (b), (e), (f) = 100 µm, (c), (d) = 250 µm.

Calcein labelling was present at both surfaces in all control and flight group bats (Fig. 1a, b, c). At the periosteal surface Ps MS/BS averaged 0.4 % in control bats and 3.3 % in the flight group. The endosteal surface was more active with Ec BS/MS mean values of 18.2 % and 17.8 % in the control and flight groups respectively.

Xylenol labelling (Fig. 1b) showed a similar trend with low Ps MS/BS (1.1 % and 0.7 %) and higher Ec MS/BS (25.9 % and 10.8 %).

The differences between Ps MS/BS of control *versus* flight groups were not significant at any time point. Ec MS/BS in control bats was significantly less than in either the flight or baseline groups for alizarin. The only significant change in amount of labelling with time was in this control group, with the (lack of) alizarin label being significantly different to either calcein or xylenol labelling.

A mineral apposition rate of 0.67 ± 0.498 μm.day^{-1} (n = 22) was determined for bats where two or more labels were visible in the same region of a single cross section. There were no discernable differences between baseline, control or flight group MAR for any treatment period.

Bone formation rates where measurable, were highly variable within and between animals. Usable data were derived predominantly from regions of endosteal bone apposition, yielding a mean value of 74.03 ± 14.84 μm^3.μm^{-2}.yr^{-1} (range = 2.52 – 277.5 μm^3.μm^{-2}.yr^{-1}, n = 22) at these active surfaces.

The small amount of new periosteal bone was added predominantly to the ventral surface, but endosteal apposition was not concentrated at any particular site. Active intracortical remodelling in the radius was limited to two (flight-group) bats. Each individual had a single labelled secondary osteon indicative of the bone formation phase of remodelling

Metacarpal III:

Bone labelling followed a similar pattern to that seen in the radii of each group, although labels were judged qualitatively to be of lower intensity than in the radii. Only three bats exhibited periosteal surface bone apposition, (Ps MS/BS = 3.2 % ± 1.3 %), and the only BFR that could be calculated was 51.1 μm^3/μm^2/yr for single flight-group animal. Endocortical surfaces were more active with the five bats exhibiting labelling (three control, one flight and one baseline animal). They had an Ec MS/BS of 17.4 ± 3.27 %, MAR of 0.86 ± 0.066 μm.day^{-1} and a maximum BFR of 216.9 μm^3.μm^{-2}.yr^{-1}.

Only two labelled secondary osteons were seen at this site (Fig. 2a, b), with both occurring in the metacarpal of a single flight-group bat.

Metacarpal V:

Low levels of bone labelling were seen (Fig. 1e), similar to those in metacarpal III. At the periosteal surface only four bats (three control and one flight animal) displayed labelling, with Ps MS/BS ranging from 0.8 % to 5.6 %. Ec MS/BS ranged from 4.8 % to 27.9 % in three bats that showed labelling. Where measurable, MAR was similar at both surfaces (0.53 ± 0.066 μm.day^{-1}, n = 6), with a mean BFR of 67.77 ± 19.36 μm^3.μm^{-2}.yr^{-1} (range: 31.7 – 106.0 μm^3.μm^{-2}.yr^{-1}, n = 4). No actively filling secondary osteons were present in any bat.

Proximal phalanx digit V:

Labelling was only present in four bats, with all labels seen at the endocortical surface. Each of the four bats displayed a single label, two with alizarin and two with xylenol. Ec MS/BS ranged from 3.1 % to 45.1 % in these bats with labels. A MAR of 0.31 μm.day^{-1} was determined from the alizarin label in two bats. There was no evidence of active or previous intracortical remodelling.

The small amounts of bone apposition that occurred during this study were not sufficient to significantly alter the polar moment of area, second moment of area and cortical thickness of any measured skeletal element.

Muscle Masses

Mean muscle masses (absolute or relative to body mass) did not differ significantly between the groups for any of the 41 muscles examined.

Bone Density – Wild bats

The skeletal elements of the forelimb exhibited considerable variation in density (≈ degree of mineralisation). The humerus and radius had the highest density at about 2 g.cm^{-3}, similar to that of the femur. Bone density declined within each digit in a proximal to distal direction, and skeletal elements in the fourth digit were of a slightly lower density than those of the third digit although the difference was not significant (Figure 3).

Discussion

A lack of measurable effect of exercise on the size of muscles associated with flight was unexpected. The observed increased in flight competence was presumably achieved by improvement in motor control and coordination, although a modification of muscle fibre type cannot be discounted. While exercise is generally considered to result in increased muscle mass, there are few data concerning flying animals. In the European starling, *Sturnus vulgaris*, increased flight-muscle exercise resulted in a small but significant decrease in body mass *and* pectoralis muscle mass (Swaddle & Biewener, 2000). Similarly, pectoral muscle thickness was found to vary in parallel with body mass in red knot, *Calidris canutus*, under flight-exercise, fasting and fuelling conditions (Lindstrom *et al*., 2000). These observations support the idea that to maintain a certain level of flight capacity pectoral muscle mass and body mass should change in parallel (Pennycuick, 1975). The small reduction in body mass and lack of significant change in muscle masses (absolute or in proportion to M_B) noted in the flight group in current study further emphasises that increases in muscle and body mass beyond that required for normal flight may be undesirable in flying animals.

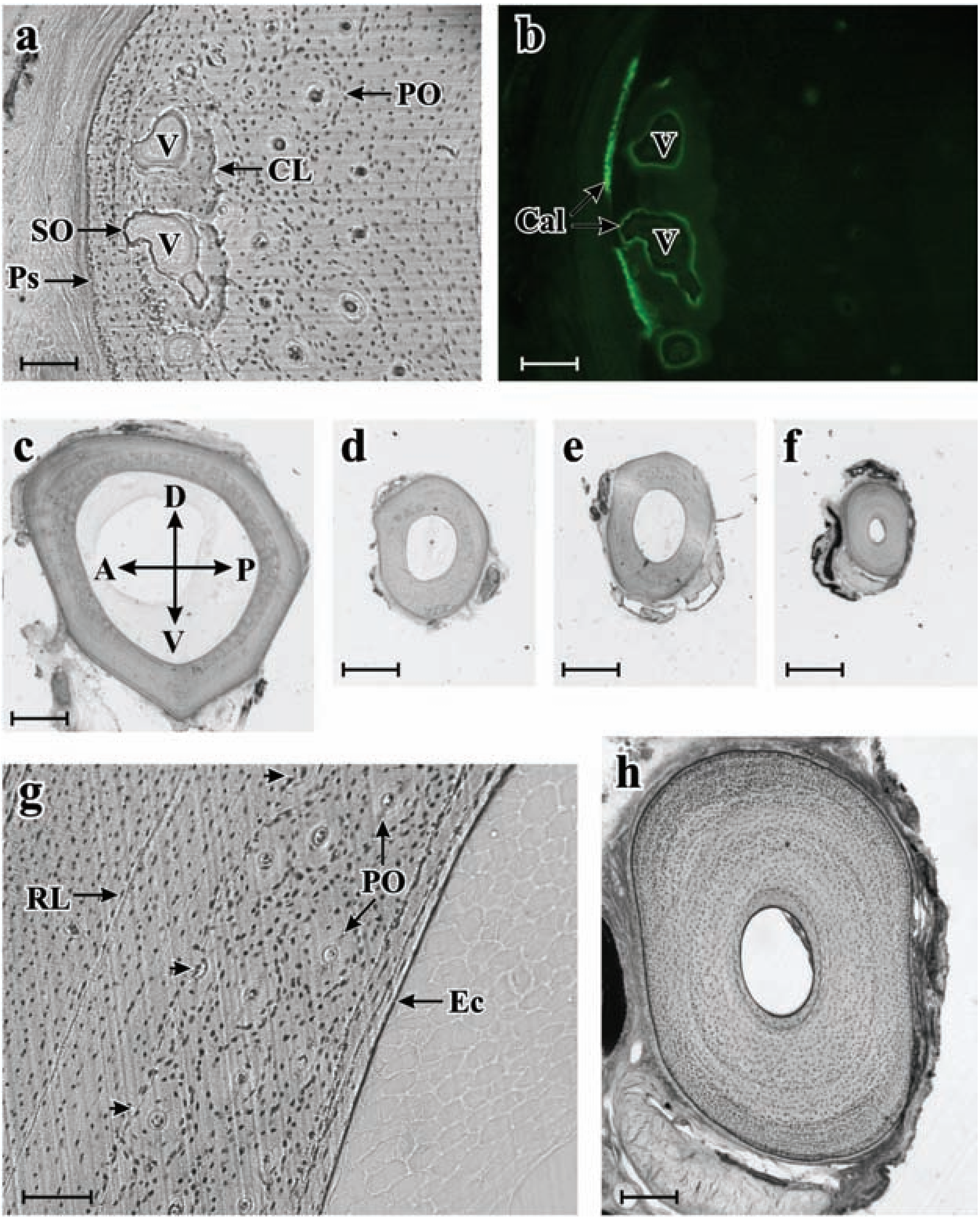

Figure 2. Mid-shaft transverse sections of undecalcified bones from a single individual. (a) Light and (b) fluorescence micrographs of metacarpal III indicating calcein labelling at the periosteum (Ps), and of the surfaces surrounding the tissue voids (V) of secondary osteons (SO). Primary osteons (PO) do not have a cement line (CL). Scale bars = 100 µm. (c) Cross-sectional morphology of the radius [with bone orientation in outstretched wing position: D, dorsal, V, ventral, L, leading, T, trailing], (d) metacarpal III, (e) metacarpal V and (f) proximal phalanx of digit V. Scale bars = 1000 µm. (g) Unstained, undecalcified section of the endocortical region of a radius. Multiple reversal lines (RL) or lines of arrest are visible. Arrows indicate where periosteal retraction has eroded pre-existing primary osteons (PO). (Scale bar = 100 µm). (h) Proximal phalanx of digit V showing a total absence of primary and secondary osteons. (Scale bar = 250 µm).

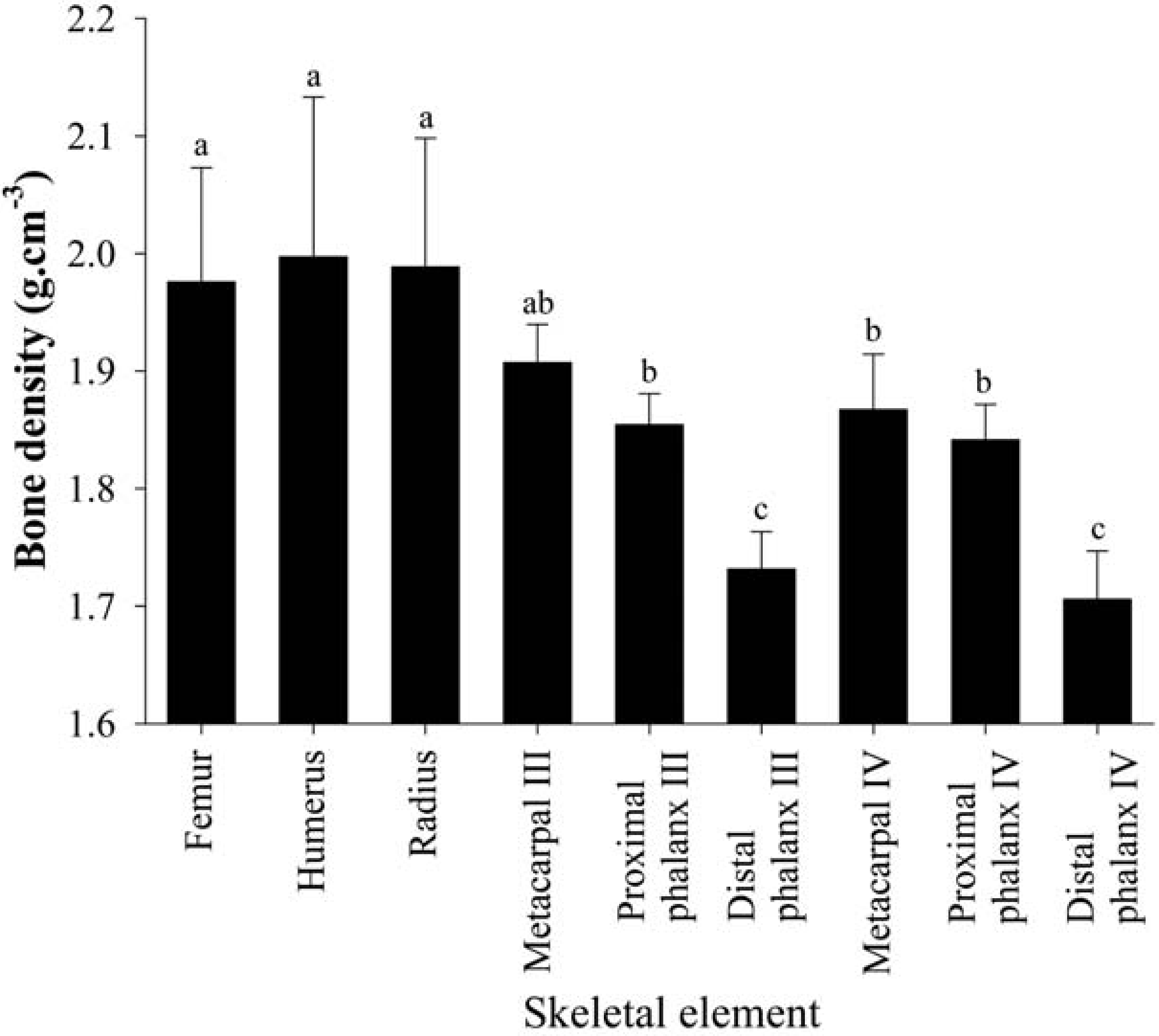

Figure 3. Density of bones from wild caught *Pteropus policephalus* determined by Archimedes' principle. Data are mean and standard deviation (n = 7). Skeletal elements that share a common letter are not statistically different ($P > 0.05$).

Bone strains of about 2,500 microstrain (μξ) were measured in the radius and humerus of free-flying *P. poliocephalus* (Swartz *et al.*, 1992) under flight conditions identical to those used in the current study. A periosteal bone strain of this magnitude, measured at the surface of the mid-shaft, corresponds to a calculated endocortical strain of about 1,675 μξ assuming bones bend symmetrically about their longitudinal axis. Strains of a similar magnitude to these (1,688 μξ) produce large and significant elevations of the bone forming surface, formation rate and mineral apposition rate at the endosteal surface in rat tibiae (Turner *et al.*, 1994). In rat tibia there appears to be a threshold of about 1,050 μξ, that when exceeded induces lamellar bone formation at the endocortical surface. A single bout of 36 cycles of sine wave loading at two hertz is sufficient to elicit bone formation (Forwood & Turner, 1994; Turner *et al.*, 1994; Forwood *et al.*, 1996). Similarly, the mechanical loading of the turkey ulna for 36 cycles per day was sufficient to elevate rates of bone formation when strains were above about 1,000 μξ (Rubin & Lanyon, 1985). Increasing the number of cycles to 1,800 per day did not potentiate the effect and as few as four cycles per day prevented bone loss. In contrast, 120 cycles per day for 194 days at strain magnitudes of about 1,700 μξ (endosteal) and 2,500 μξ (periosteal) did not produce significant bone formation in the present study.

Active intracortical remodelling was infrequent in our study animals; being observed in only three of the 44 bones studied. Schaffler & Burr's (1984) data from limb bones of primates suggest that the amount of intracortical remodelling is related to the loading environment typically encountered - the greater the degree of load-bearing use a bone is put to the greater the remodelling. Intracortical remodelling also appears to be common in the furculae of birds, although the determinants of the amount of remodelling remain to be resolved (Ponton *et al.*, 2007). While it has been argued that intracortical remodelling is linked predominantly to the repair of fatigue damage (Mori & Burr, 1993; Pattin *et al.*, 1996; Frost, 1997; Bentolila *et al.* 1998) it also occurs in situations of chronic disuse (Rubin & Lanyon, 1984). The relative lack of active intracortical remodelling in the present study suggested that the bats' bones did not fall into this disuse category, and that the repetitive strains imposed by active flight were insufficient to initiate intracortical remodelling (Burr *et al.*, 1985).

With the exception of proximal phalanx V, secondary remodelled (Haversian) bone was seen in cross sections of all bones. Its location, mainly on the inner portion of the cross-section (Figs. 1c, f, 2c, d, e, g), coupled with the fact that it was predominantly quiescent, suggested that remodelling had occurred in an early stage of life, possibly when the wings were being exercised in preparation for anticipated flight. The lack of intracortical remodelling in the phalanges of digit V in any of the bats was interesting. It raises questions about how fatigue-induced microdamage accumulation is avoided or repaired in the distal wing bones of bats. It is especially pertinent as strains of about 5,000 μξ have been measured in distal wing bones of *P. poliocephalus* during flight (unpub. obs.) and at this level of strain the time to structural failure of bone is reported to be as little as about 12 seconds (see Ziopous *et al.* 2001). In wild bats these bones are subjected to thousands of cyclical-loading events per day for many years and it may be that the relatively low level of mineralisation of distal bones, shown in figure 3, provides them with fatigue-resistant properties. Our observations that fluorochrome labels in distal wing bones were of much lower intensity when compared to those in the radius, is consistent with a lower mineralisation at these sites The pattern of reduced bone density in a proximal to distal direction (Fig. 3) may result in energetic benefits by reducing the mass of the distal wing that has to be repeatedly accelerated and decelerated during flight. Wing bone stiffness could be maintained by developing an oval cross-section (Fig. 2d, e, f) with the greater diameter aligned with the direction of maximum bending force encountered during flight.

The move from the original holding facility to the small (8 m^3) cage resulted in resorption of bone from the endosteal surface in the non-exercised group, indicative of 'disuse' endosteal remodelling, prior to a period of modest bone apposition. Presumably, the flight group avoided a similar resorption event due to their exercise regimen.

Swartz *et al.* (1992) argued that actively flying vertebrates have large diameter, thin-walled wing bones to maximise the polar moment of inertia of the bones for a given volume (or mass) of bone. This would maximise torsional stiffness and strength to withstand the relatively large torsional stresses that act during the wing beat cycle. A similar argument, but based on bending strengths, was proffered by van der Meulen *et al.*(1992) in their analysis of limb bone strengths in pterosaurs. The initial hypothesis of the present study proposed that a periosteal expansion would accompany the flight-training and exercise regimen - increasing torsional stiffness and strength of wing bones. This hypothesis could not be supported by our results. The small net deposition of bone at the endosteal surface in our exercised bats was unexpected. This appears to be a mechanically inappropriate site for bone formation, as it is relatively close to the axes of bending or torsion, and will therefore have relatively little influence on bending or torsional bone stiffness and strength. The 'lack' of periosteal bone deposition was also unexpected. There are a number of possible explanations for this result. One is that the strain magnitude (c. 2,500 μξ) was insufficient to engender an anabolic response, although lower strains in other mammals have been shown to be osteogenic. If so, then perhaps the strain threshold for bone formation is high in this species at these sites. That is, the strain threshold for adaptive modelling at specific skeletal sites may be genetically determined in relation to the strain requirements for flight. Hence, it may be that this species of bat develops a wing skeleton of sufficient mechanical strength for the demands of active flight, even in the absence of normal flight behaviour. As such, although conventional wisdom is that exercise impacts on skeletal remodelling it is possible that certain species may have little scope or lack phenotypic plasticity, particularly if there is little natural variation in activity.

Alternatively, as has been seen in other skeletally mature mammals, it may be that the scope for a periosteal appositional response to increased bone strains was very limited (Forwood & Burr, 1993, Parfitt, 1994; Khan *et al.*, 2000, Forwood, 2008). For example, the dominant hands and forearms of tennis players undergo greater hypertrophy than the non-dominant limb (Jones *et al.*, 1977; Kannus *et al.*, 1995). However, when adjusted for the age at which racquet sports were begun, side to side differences in bone mineral content of the humerus was two to four times greater in female players who started training before or at menarche compared to those who started 15 years after menarche (Kannus *et al.*, 1995). Such data add to the evidence that the consequence of physical activity with regard to the adult skeleton is conservation, not acquisition (Forwood & Burr, 1993; Parfitt 1994). The results from the current study are consistent with this hypothesis.

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